Evidence for Kynurenine in Milk

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Abstract

Kynurenine, a potential precursor to o-aminoacetophenone in dry and sterile concentrated milks, was found to be a constituent of fresh milk. Employing alkaline degradation studies on the basic fraction of milk serum, obtained by negative pressure dialysis and ion exchange extraction, concentration of kynurenine was found to be in the order of one μM per liter of raw milk serum. Evidence is presented to show that the concentration of kynurenine in milk serum decreases as the original milk is heated at temperatures exceeding 93.3 C for 15 sec.

A previous investigation (3) in this laboratory has implicated o-aminoacetophenone as an off-flavor component of stale dry and sterile concentrated milks. In that report, it was suggested that the flavor compound could conceivably arise during storage from tryptophan, indican (indoxyl sulfuric acid), or kynurenine. Of the three potential precursors to o-aminoacetophenone, kynurenine has not, to our knowledge, been, reported as a constituent of milk. This study describes our efforts to establish the presence of kynurenine in fresh milk.

Experimental Procedure[1]

Milk sera.[2] Milk sera were obtained by negative-pressure dialysis of raw or heated skimmilk in the following manner: The milk was fed from a separatory funnel attached at the stem to 60 cm of size 8/100-ft dialysis tubing (Union Carbide Corporation, Food Products Division, 6733 W. 65th Street, Chicago, Illinois) held under vacuum in a one-liter suction flask. Dialysis was allowed to proceed for 18 hr at 2.2 C. Where employed, skimmilk (obtained from mixed-herd milk, Beltsville, Maryland) was heated at temperatures up to 143.3 C for 15 sec in a Mallory heater.

Ion-exchange extraction of the basic fraction of milk serum. One hundred milliliters of milk serum was passed over 5 g (wet weight) of Dowex 50–8X$^{(H+)}$ at a flow rate of 1–2 ml per minute. The column was washed with 100 ml of distilled water and the basic fraction eluted with 20 ml of 5 N NH_4OH. The effluent was collected in a 200-ml round-bottomed flask and taken to dryness on a rotary film evaporator under vacuum.

Kynurenine and tryptophan determinations. The kynurenine content of milk was determined by a slight modification of the method of Spacek (5), which depends upon the alkaline degradation of kynurenine to o-aminoacetophenone. The residue (basic fraction) obtained from evaporation of the 5 N NH_4OH effluent was reconstituted with 100 ml of distilled water with the aid of slight heat and transferred to a two-liter round-bottomed flask. To the flask was added 15 g of solid sodium hydroxide and a few boiling chips. The mixture was distilled on a heating mantle controlled by a variable transformer. For comparative studies, the mixture was distilled at the same rate. This was accomplished by employing the same variable transformer setting (75) at all times. The first 50 ml of distillate, condensed with a short Friedrich condenser, was collected and extracted with 10 ml of chloroform. The chloroform extract was dried with sodium sulfate and the optical density obtained with a Beckman DU spectrophotometer at 358 mμ, the wave length of maximum absorption of o-aminoacetophenone in chloroform. Preliminary studies revealed that o-aminoacetophenone has a molar extinction coefficient of 4,850 in chloroform at the wave length of maximum absorption. Molar and weight concentrations of kynurenine in milk serum were determined by calculations from the optical density readings and molar extinction coefficient of o-aminoacetophenone.

Tryptophan determinations on the basic fraction were made by the method of Spies and Chambers (6).

Gas-liquid chromatography. GLC studies were performed on a Research Specialties' instrument equipped with an argon ionization detector employing Sr^{90} as the radioactive source. Chromatographic conditions included a 180- by 0.63-cm stainless steel column packed with 10% Apiezon L on a 60–80-mesh-silaned celite operated at a temperature of 120 C and 20 psi argon pressure.

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[1] Reference to certain products or companies does not imply an endorsement by the Department over others not mentioned.

[2] Milk serum as used here refers to the dialysate obtained by negative-pressure dialysis.

Thin-layer chromatography. Separation of the basic fraction was accomplished by TLC on Silica Gel G. The chromatograms were developed with tert-butanol: methylethyl ketone: water: ammonium hydroxide (40:30:20:10), and the spots made visible with a ninhydrin-cupric nitrate indicator (1).

Results and Discussion

According to Spacek (5), the alkaline degradation of kynurenine results in a > 90% yield of o-aminoacetophenone, a figure with which we are in agreement as a result of our preliminary studies. Employing the alkaline degradation procedure to the basic fraction of milk serum obtained by ion-exchange, an average concentration of 1 μM of o-aminoacetophenone per liter of raw milk serum was obtained. Evidence that o-aminoacetophenone was present in the distillate was readily obtained by its characteristic odor and verified by GLC retention-time studies.

The specificity of the method for determining kynurenine in urine has been dealt with by Spacek (4, 5) and the conclusions reached in those studies appear applicable to milk serum. Of the known constituents of milk serum, tryptophan is most likely to interefer with quantitative determinations of kynurenine, employing Spacek's method. However, his results indicate, and we are in agreement, that the yield of o-aminoacetophenone from tryptophan by alkaline degradation is less than 0.1%. Tryptophan determinations on the basic fraction from milk serum resulted in concentrations equivalent to 1–2 μM of tryptophan per liter of serum. It can be concluded, therefore, that tryptophan accounts for an insignificant amount of o-aminoacetophenone arising in these studies.

Confirming evidence for the presence of kynurenine in milk was obtained by heated-milk studies in conjunction with TLC studies. We had noticed early in our studies (2) that, as milk was heated above 93.3 C for 15 sec, the concentration of o-aminoacetophenone obtained from the basic fraction of such milks decreased drastically. Results reported in Table 1 for one series of samples are quite typical of many such determinations, in that a slight increase in o-aminoacetophenone results in milk sera from skimmilk heated at 76.7 C for 15 sec over that of unheated milk, followed by a decrease in concentration at higher heating temperatures.

That kynurenine is present in the basic fraction was further demonstrated by TLC. A spot which moved like kynurenine and had the same color characteristic (orange) when developed with the ninhydrin–cupric nitrate spray, yielded o-aminoacetophenone when extracted from a series of plates and subjected to alkaline degradation. Quantitative TLC of the basic fraction from milk sera obtained from raw and heated milks revealed a decrease in the intensity of the kynurenine spot from milks heated at 126.7 C and the complete absence of the kynurenine spot in milks heated at 143.3 C for 15 sec. Hence, the visible observation of a decrease in the kynurenine content parallels the loss of o-aminoacetophenone arising by alkaline degradation.

TABLE 1

Kynurenine content of the basic fraction of milk. Sera obtained from skimmilk heated at various temperatures

Sample	μM of o-Aminoacetophenone per 100 ml of serum	μg of Kynurenine per liter of milk serum
Raw	0.113	235.0
76.7 C, 15 sec	0.118	245.0
93.3 C, 15 sec	0.113	235.0
110.0 C, 15 sec	0.080	166.4
126.7 C, 15 sec	0.036	74.9
143.3 C, 15 sec	0.010	20.8

Although the evidence presented herein confirms our belief that kynurenine is present in milk, it does not implicate this tryptophan metabolite as the precursor of o-aminoacetophenone in dry and sterile concentrated milks. Studies to clarify the role of kynurenine in the off-flavors of these products, in light of its apparent loss during heat treatment of milk, are under investigation at the present time.

References

(1) Moffat, E. O., and Lytle, R. I. 1959. Polychromatic Technique for the Identification of Amino Acids on Paper Chromatograms. Anal. Chem., 31:926.

(2) Parks, O. W. 1965. The Flavor of Milk. Presented Sympo. on Foods: Chemistry and Physiology of Flavor. Oregon State University, Corvallis. September 8–10.

(3) Parks, O. W., Schwartz, D. P., and Keeney, M. 1964. Identification of o-Aminoacetophenone as a Flavour Compound in Stale Dry Milk. Nature, 202:185.

(4) Spacek, M. 1953. Tryptophane Metabolites in Human Urine. Nature, 172:204.

(5) Spacek, M. 1954. Simultaneous Determination of Kynurenine and p-Phenetidine in Human Urine. Canadian J. Biochem. Physiol., 32: 604.

(6) Spies, J. R., and Chambers, D. C. 1948. Chemical Determination of Tryptophan. Anal. Chem., 20:30.